APPLIED AND ENVIRONMENTAL MICROBIOLOGY, July 1992, p. 2334–2337
0099-2240/92/072334-04$02.00/0

Vol. 58, No. 7

Roles of Arginine in Growth of *Clostridium botulinum* Okra B

SANDRA I. PATTERSON-CURTIS[1]† AND ERIC A. JOHNSON[1,2]*

Departments of Food Microbiology and Toxicology[1] and Bacteriology,[2] University of Wisconsin, 1925 Willow Drive, Madison, Wisconsin 53706

Received 15 January 1992/Accepted 25 April 1992

Group I strains of *Clostridium botulinum* are known to degrade arginine by the arginine deiminase pathway. We have found that *C. botulinum* Okra B consumed a level of arginine (20 g/liter) higher than the basal requirement for growth (3 g/liter). Arginine was probably the preferred source of nitrogen for biosynthesis but did not serve as a major source of energy. Citrulline and proline were produced as major fermentation products in media containing high levels of arginine, but in media with basal amounts of arginine these products were produced in lower quantities during growth and were later reassimilated. The results indicate that *C. botulinum* Okra B changes its metabolism during consumption of surplus arginine, and this change is associated with toxin repression, formation of citrulline and proline as end products, and possibly resistance to environmental stresses such as increased acidity and osmolarity.

Early studies of the nutrition of *Clostridium botulinum* demonstrated a requirement for arginine in proteolytic strains that produce type A or B neurotoxin (8, 12, 19). *C. botulinum* and the closely related organism *Clostridium sporogenes* degrade arginine by the arginine deiminase (ADI) pathway to citrulline which is further converted to ornithine and proline (1, 5, 15) and probably to glutamate (1, 4). *C. botulinum* and *C. sporogenes* appear to differ slightly in metabolism of arginine (8, 22, 24). In *C. sporogenes*, arginine can support growth in the absence of sugar (24). *C. botulinum* also prefers arginine in a mixture of nutrients (23), but substantial growth requires glucose or another sugar as a source of energy.

During the study of regulation of neurotoxin formation in *C. botulinum* by arginine (17, 18), we observed that the Okra B strain consumed quantities of arginine well in excess of the growth requirement. Degradation of high levels of arginine was of particular interest since arginine is probably the limiting organic nutrient for growth of group I *C. botulinum* in many foods and media (23) and because protease and neurotoxin production are repressed by high levels of arginine (17, 18). Because basic end products and proline are formed during arginine fermentation, catabolism of high levels of arginine could provide protection against environmental stresses, including increased acidity (3, 21) and osmolarity (6, 14). In this study, we studied the digestion of surplus levels of arginine by *C. botulinum* Okra B.

To study arginine metabolism, Okra B was grown in a chemically defined minimal medium (MI) as previously described (17, 18, 23). MI contains a basal level of arginine (3 g/liter) that supports growth measured at A_{660} to ~1 (1.08 mg [dry cell weight] per ml) (23). This basal requirement of 3 g/liter is more than the expected level needed for protein biosynthesis (~100 mg/liter), suggesting that arginine is used as a primary source of energy, carbon, or nitrogen. Additional experiments showed that *C. botulinum* Okra B digested 20 g of arginine per liter (17), which is 6-fold higher than the basal requirement and nearly 200-fold the expected level required for biosynthesis. We investigated catabolism of arginine provided at the basal requirement (3 g/liter) and at a high level (20 g/liter).

C. botulinum Okra B had similar patterns of growth in MI containing either a basal (3 g/liter) or high (20 g/liter) level of arginine (Fig. 1A), but lysis was slightly delayed with the high level of arginine as was observed earlier (2). Arginine consumption differed between basal and high levels of arginine (Fig. 1B). When supplied at 3 g/liter, nearly all the arginine (2.9 g/liter) was consumed during exponential growth (<24 h). When arginine was provided at 20 g/liter, about 10 g/liter was used during exponential growth, and it continued to be used until 19.2 g/liter was depleted by the end of the stationary phase and the start of lysis (81 h).

Consumption of high levels of arginine suggested that arginine may provide carbon and nitrogen for energy or biosynthesis and possibly other functions. In media containing a high level of arginine, glucose consumption was decreased from 8.5 to 7.5 g/liter, indicating that arginine could partially replace glucose and provide energy. Very little growth occurred when glucose was omitted from MI or when ornithine replaced arginine in the absence of glucose. These results indicated that arginine may have provided relatively small quantities of energy but that glucose was the major source of energy. We tested whether cell extracts of *C. botulinum* could use carbamoylphosphate as a phosphoryl donor for glucose, as occurs in *Streptococcus faecalis* (16). Phosphorylation to glucose 6-phosphate was detected with ATP as a phosphoryl donor but not with carbamoylphosphate, acetylphosphate, or phosphoenolpyruvate (data not shown). These results indicated that arginine was not directly involved in the phosphorylation of sugars in glycolysis in *C. botulinum* Okra B.

We next determined whether the arginine consumed by the cells was incorporated into cell mass and whether products of arginine digestion were released into the medium. Growth of *C. botulinum* in media containing 2 or 13 μCi of L-[U-^{14}C]arginine (>300 mCi/mmol) and unlabeled arginine at 3 or 20 g/liter showed that only a small percentage of label (<20%) was incorporated into cell mass. The majority of the label was released as unidentified extracellular products. Various soluble nitrogenous catabolites of the ADI pathway released into the medium were determined as described previously (17, 18). As observed earlier (18), ammonia liberation from arginine was negligible. However,

* Corresponding author.
† Present address: Quest International, Microlife Technics, Sarasota, FL 34230.

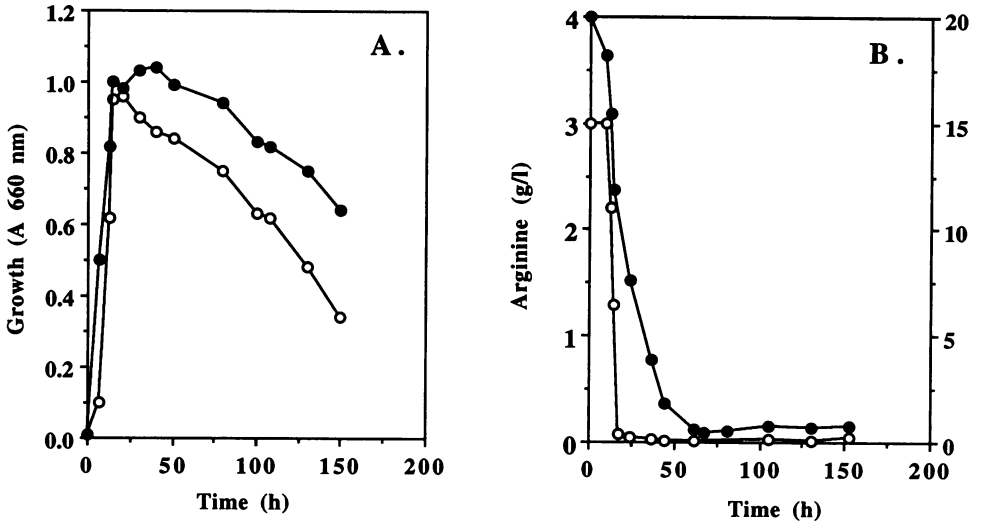

FIG. 1. Growth and lysis (A) and arginine utilization (B) by *C. botulinum* in media containing a basal (3 g/liter [○]) or high (20 g/liter [●]) level of arginine. Media contained 1% glucose.

substantial quantities of citrulline and proline were detected as extracellular products during the growth cycle. In MI containing a basal level of arginine, about 1 g of citrulline per liter was released from the cells early in growth and was reassimilated during continued exponential growth (Fig. 2A). In MI containing 20 g of arginine per liter, about 5 g of citrulline per liter was released in the exponential phase (Fig. 2B), but it was not reassimilated by the cells. Arginine was depleted from the medium at the same time that citrulline reached its maximum level. Small quantities of ornithine were also released extracellularly (0.18 and 1.6 g/liter in basal and high levels of arginine, respectively), which was completely scavenged by 50 h in basal and high levels of arginine (data not shown).

Different patterns of proline formation and uptake were observed with basal and high levels of arginine. With the basal level of arginine, 0.7 g of proline per liter (equal to that derived from 1.05 g of arginine) was released extracellularly by the end of arginine depletion and was later assimilated (Fig. 3A). 5-Aminovalerate (0.78 g/liter) formed by reduction of proline was detected in the culture fluid from stationary-phase cells. With the high level of arginine, 6.9 g of proline per liter (equal to that derived from 10.5 g of arginine per liter) was detected in growth and stationary phases (Fig. 3B), but the proline was not later assimilated. Most of the proline was released after arginine depletion from the medium, and only 0.30 g of 5-aminovalerate per liter was detected in the cultures with 20 g of arginine per liter.

The results of this study indicate that *C. botulinum* prefers arginine over ammonia or other amino acids as a source of nitrogen for biosynthesis. Arginine metabolism in *C. botulinum* appears unusual compared with that in other organisms that possess the ADI pathway. Arginine does not appear to be a major source of energy in *C. botulinum* Okra B. *C.*

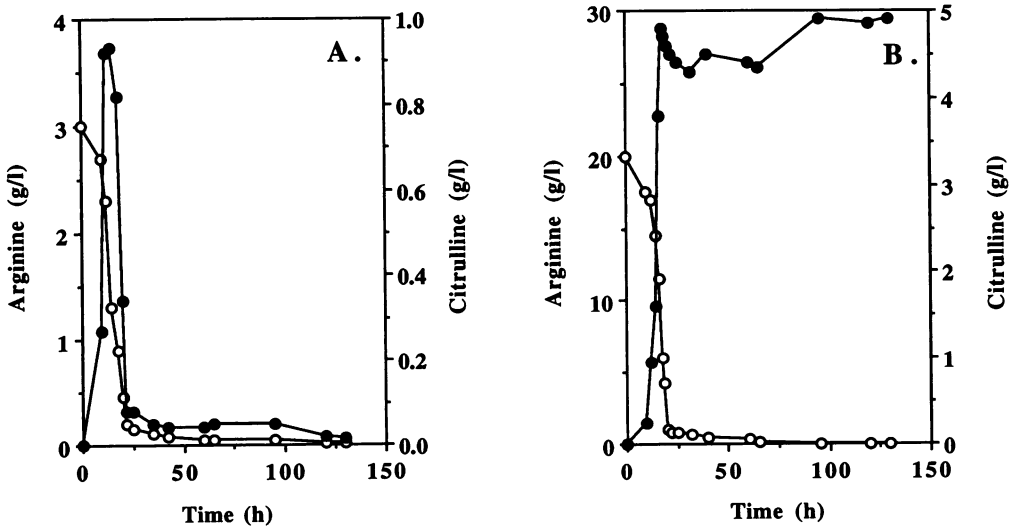

FIG. 2. Utilization of arginine (○) and formation and reassimilation of citrulline (●) by *C. botulinum* in a basal (3 g/liter [A]) or high (20 g/liter [B]) level of arginine.

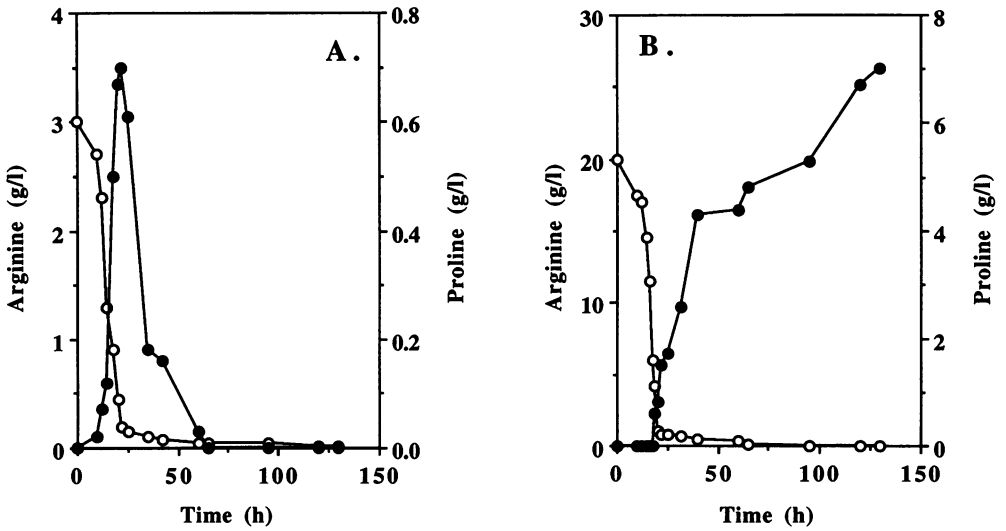

FIG. 3. Utilization of arginine (○) and formation and reassimilation of proline (●) by *C. botulinum* in a basal (3 g/liter [A]) or high (20 g/liter [B]) level of arginine.

botulinum produces extracellular citrulline and proline when arginine is available in the medium but reassimilates these products when arginine is depleted. Many bacteria with the ADI pathway excrete 1 mol of ornithine per mol of arginine metabolized (7, 10, 20), although mutants that excrete citrulline instead of ornithine have been characterized (9, 25). In lactococci, arginine metabolism by the ADI pathway is directly linked to membrane transport by the antiport of arginine and ornithine (10, 13). It appears that in *C. botulinum* arginine uptake may be coupled to antiport of citrulline. During this exchange the cells may also accomplish the extrusion of protons or sodium ions (11, 13). This general utility of arginine in membrane processes could explain the property of the pathogen to consume much higher levels of arginine than are required for growth.

We showed earlier that growth and toxin formation are controlled by arginine (17, 18, 23). In the present study, we have found that during metabolism of surplus arginine, *C. botulinum* Okra B accumulates basic end products such citrulline and proline, which could help protect the organism from environmental stresses, including acidity and osmolarity. Our results support the hypothesis that the level of arginine in foods or in the human intestinal tract is critical for growth, survival, and toxin formation by *C. botulinum*.

This work was supported by a Hatch Grant from the University of Wisconsin Agricultural Experiment Station, by the Center for Dairy Research, College of Agricultural and Life Sciences of the University of Wisconsin—Madison, and by the Wisconsin Milk Marketing Board.

We thank Greg Leyer for helpful comments on the manuscript.

REFERENCES

1. **Barker, H. A.** 1981. Amino acid degradation by anaerobic bacteria. Annu. Rev. Biochem. **50:**23–40.
2. **Bowers, L. E., and O. B. Williams.** 1963. Effect of arginine on growth and lysis of *Clostridium botulinum*. J. Bacteriol. **85:** 1175–1176.
3. **Casiano-Colón, A., and R. E. Marquis.** 1988. Role of the arginine deiminase system in protecting oral bacteria and an enzymatic basis for acid tolerance. Appl. Environ. Microbiol. **54:**1318–1324.
4. **Costilow, R. N., and D. Cooper.** 1978. Identity of proline dehydrogenase and Δ^1-pyrolline-5-carboxylic acid reductase in *Clostridium sporogenes*. J. Bacteriol. **134:**139–146.
5. **Costilow, R. N., and L. Laycock.** 1969. Reactions involved in the conversion of ornithine to proline in clostridia. J. Bacteriol. **100:**662–667.
6. **Csonka, L. N.** 1989. Physiological and genetic responses of bacteria to osmotic stress. Microbiol. Rev. **53:**121–147.
7. **Cunin, R., N. Glansforff, A. Pierard, and V. Stalon.** 1986. Biosynthesis and metabolism of arginine in bacteria. Microbiol. Rev. **50:**314–352.
8. **Elberg, S. S., and K. F. Meyer.** 1939. The nutritional requirements of *Clostridium parabotulinum*, A. J. Bacteriol. **37:**429–445.
9. **Fenske, J. D., and G. E. Kenny.** 1976. Role of arginine deiminase in growth of *Mycoplasma hominis*. J. Bacteriol. **126:**501–510.
10. **Konings, W. N., B. Poolman, and A. J. M. Driessen.** 1989. Bioenergetics and solute transport in lactococci. Crit. Rev. Microbiol. **16:**419–476.
11. **Lovitt, R. W., D. B. Kell, and J. G. Morris.** 1986. Proline reduction by *Clostridium sporogenes* coupled to vectorial proton ejection. FEMS Microbiol. Lett. **36:**269–273.
12. **Mager, J., S. H. Kindler, and N. Grossowicz.** 1954. Nutritional studies with *Clostridium parabotulinum* type A. J. Gen. Microbiol. **10:**130–141.
13. **Maloney, P. C.** 1983. Relationship between phosphorylation potential and electrochemical H^+ gradient during glycolysis in *Streptococcus lactis*. J. Bacteriol. **153:**1461–1470.
14. **Measures, J. C.** 1975. Role of amino acids in osmoregulation of non-halophilic bacteria. Nature (London) **257:**398–400.
15. **Mitruka, B. M., and R. N. Costilow.** 1967. Arginine and ornithine catabolism by *Clostridium botulinum*. J. Bacteriol. **93:** 295–301.
16. **Pandey, V. N.** 1980. Interdependence of glucose and arginine catabolism in *Streptococcus faecalis* ATCC 8043. Biochem. Biophys. Res. Commun. **96:**1480–1487.
17. **Patterson-Curtis, S.** 1990. Ph.D. thesis. University of Wisconsin, Madison.
18. **Patterson-Curtis, S. I., and E. A. Johnson.** 1989. Regulation of neurotoxin and protease formation in *Clostridium botulinum* Okra B and Hall A by arginine. Appl. Environ. Microbiol. **55:**1544–1548.
19. **Perkins, W. F., and K. Tsuji.** 1962. Sporulation of *Clostridium botulinum*. II. Effect of arginine and its degradation products on sporulation in synthetic medium. J. Bacteriol. **84:**86–94.
20. **Stalon, V.** 1985. Evolution of arginine metabolism, p. 277–308. *In* K. H. Schleifer and E. Stackebrandt (ed.), Evolution of

prokaryotes. FEMS Symposium no. 29. Academic Press, Inc., London.
21. **Thomas, T. D., and R. D. Batt.** 1969. Metabolism of exogenous arginine and glucose by starved *Streptococcus lactis* in relation to survival. J. Gen. Microbiol. **58:**371–380.
22. **Venugopal, V., and G. B. Nadkarni.** 1977. Regulation of the arginine dihydrolase pathway in *Clostridium sporogenes*. J. Bacteriol. **131:**693–695.
23. **Whitmer, M. E., and E. A. Johnson.** 1988. Development of improved defined media for *Clostridium botulinum* serotypes A, B, and E. Appl. Environ. Microbiol. **54:**753–759.
24. **Wildenauer, F. X., and J. Winter.** 1986. Fermentation of isoleucine and arginine by pure and syntrophic cultures of *Clostridium sporogenes*. FEMS Microbiol. Ecol. **38:**373–379.
25. **Yamamoto, K., T. Sato, T. Tosa, and I. Chibata.** 1974. Continuous production of L-citrulline by immobilized *Pseudomonas putida*. Biotechnol. Bioeng. **16:**1589–1599.